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International application number: PCT/US04/040674

International filing date: 03 December 2004 (03.12.2004)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/528,161
Filing date: 08 December 2003 (08.12.2003)

Date of receipt at the International Bureau: 31 January 2005 (31.01.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

1275160

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APPLICATION NUMBER: ***60/528,161***
FILING DATE: ***December 08, 2003***
RELATED PCT APPLICATION NUMBER: ***PCT/US04/40674***

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PROVISIONAL APPLICATION FOR PATENT
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Commissioner for Patents
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ATTENTION: PROVISIONAL PATENT APPLICATION

Sir:

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR § 1.53(c).

For: **IDENTIFICATION OF CHIMPANZEE FAB FRAGMENTS BY REPERTOIRE CLONING AND PRODUTION OF A FULL-LENGTH HUMANIZED IgG1 ANTIBODY HIGHLY EFFICIENT FOR NEUTRALIZATION OF DENGUE TYPE 4 VIRUS**

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Enclosed are:

(X) Specification in 25 pages.

(X) Seven (7) sheets of drawings.

(X) Four (4) sheets of tables.

(X) ATCC Patent Deposit Receipt.

(X) A check in the amount of $160 to cover the filing fee is enclosed.

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Was this invention made by an agency of the United States Government or under a contract with an agency of the United States Government?

(X) Yes. The name of the U.S. Government agency and the Government contract number are: National Institutes of Health.

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Attorney Docket No.	:	NIH272.001PR
Applicant(s)	:	Lai et al.
For	:	**IDENTIFICATION OF CHIMPANZEE FAB FRAGMENTS BY REPERTOIRE CLONING AND PRODUTION OF A FULL-LENGTH HUMANIZED IgG1 ANTIBODY HIGHLY EFFICIENT FOR NEUTRALIZATION OF DENGUE TYPE 4 VIRUS**
Attorney	:	Nancy W. Vensko
"Express Mail" Mailing Label No.	:	EV309082668 US
Date of Deposit	:	December 8, 2003

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REFERENCE: Patent Deposit (Ref: Docket or Case No.: NIH272.001PR)

Plasmid: Humanized IgG1 5H2 assigned PTA-5662.

Date of Deposit: November 26, 2003. Paperwork will be forwarded to you in a few days. An invoice will be sent under separate cover referencing your MasterCard as follows:

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Marie Harris

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Identification of chimpanzee Fab fragments by repertoire cloning and production of a full-length humanized IgG1 antibody highly efficient for neutralization of dengue type 4 virus

Summary

A safe and effective dengue vaccine is still not available. Passive immunization using monoclonal antibodies from humans or non-human primates represents an attractive alternative for prevention of dengue. Fab monoclonal antibodies to dengue type 4 virus (DENV-4) were recovered by repertoire cloning of bone marrow mRNAs from an immune chimpanzee and analyzed for antigen binding specificity, V_H and V_L sequences and neutralizing activity against DENV-4 *in vitro*. Fabs 5A7, 3C1, 3E4 and 7G4 were isolated from a library constructed from a chimpanzee following intra-hepatic transfection with infectious DENV-4 RNA. Fabs 5H2 and 5D9, which shared a nearly identical V_H sequence, but varied in their V_L sequences, were recovered from a library constructed from the same chimpanzee after super-infection with a mixture of DENV-1, DENV-2 and DENV-3 viruses. In radio-immunoprecipitation, Fab 5A7 precipitated only DENV-4 prM and Fabs 3E4, 7G4, 5D9 and 5H2 precipitated DENV-4 E but little or no prM. Fab 3E4 and Fab 7G4 competed with each other for binding to DENV-4 in ELISA, as did Fab 3C1 and Fab 5A7. Fab 5H2 recognized an epitope on DENV-4 that was separate from the epitope(s) recognized by other Fabs. Both Fab 5H2 and Fab 5D9 neutralized DENV-4 efficiently with a titer of 0.24-0.58 μg/ml by plaque reduction neutralization test (PRNT), whereas DENV-4 neutralizing activity of other Fabs was low or not detected. Fab 5H2 was converted to full-length IgG1 by combining it with human sequences. The humanized chimpanzee antibody IgG1 5H2 produced in CHO cells neutralized DENV-4 strains from different geographical origins at a similar $PRNT_{50}$ titer of 0.03-0.05 μg/ml. The DENV-4 binding affinities were 0.42 nM for Fab 5H2 and 0.24 nM for full-length IgG1 5H2. Monoclonal antibody IgG1 5H2 may prove valuable for passive immunoprophylaxis against dengue in humans.

INTRODUCTION

Among the arthropod-borne flaviviruses, the four dengue virus serotypes (DENV-1-4) that constitute a serologically distinct subgroup are most important in terms of human morbidity and geographic distribution. Dengue viruses cause dengue outbreaks and major epidemics in most tropical and subtropical areas where *Aedes albopictus* and *Aedes aegypti* mosquitos are abundant. Dengue infection produces fever, rash, and joint pain in humans. A more severe and life-threatening form of dengue, characterized by hemorrhagic fever and hemorrhagic shock, has occurred with increasing frequency in Southeast Asia and Central and South America, where all four dengue virus serotypes circulate. The underlying cause of severe dengue remains controversial (23, 53). An association of severe dengue with increased viral replication has been reported recently (61). A safe and effective vaccine against dengue is currently not available.

The dengue virus contains a positive strand RNA genome, coding for a polyprotein that is cleaved co- and post-translationally by a combination of cellular and viral proteases to generate the individual viral proteins (9, 19, 40). Dengue virus prM and E structural proteins and nonstructural NS1 protein are glycosylated. The prM glycoprotein is further cleaved by the cellular enzyme furin following viral assembly, generating M, which is present in the mature virus (58). Flavivirus prM and E form heterodimers, which are assembled into viral particles during infection (62). In this manner, the prM serves to protect the functional integrity of E from acid-induced conformational change (26, 32). The E glycoprotein is responsible for cell attachment, possibly mediated by a receptor, and for fusion with the cell membranes following viral entry.

Mouse monoclonal antibodies against the dengue viruses have been valuable for dengue virus serotype determination (20, 27). Studies using monoclonal antibodies against dengue and other flaviviruses have also provided valuable information concerning the antigenic structure of the major viral antigen E (24, 25, 29, 39, 52). The 3-dimensional structure of the E glycoprotein has been determined at 2 ÅÅ resolution for tick-borne encephalitis virus and recently for dengue type 2 virus (45, 51). These studies showed that the monomeric E polypeptide is folded into three distinct domains and that the E glycoprotein consists of a flat elongated dimer structure with an inter-domain ligand-binding pocket.

Monoclonal antibodies reactive to flavivirus envelope proteins have been shown to mediate protection against homologous virus challenge in animal models (6, 22, 34, 35, 42). In most cases, protection by passive immunization has been correlated with the ability of these antibodies to neutralize the virus *in vitro*. Protection against dengue virus challenge was also demonstrated in mice following passive immunization with monoclonal or polyclonal antibodies specific to prM (7, 34) or NS1 (18, 28).

Most research efforts directed to the development of an attenuated live dengue vaccine have not yielded a satisfactory result. Recently, clinical evaluation was conducted on a genetically engineered DENV-4 mutant containing a 30-nucleotide deletion in the 3' non-coding region that exhibited reduced replicative capacity in simian cell culture and in primates (14, 44). Following a single dose inoculation, a total of 20 volunteers remained afebrile and exhibited very few clinical signs. Each of the vaccinees developed a high titer of DENV-4 neutralizing antibodies four to six weeks after immunization. However, five vaccinees showed an elevation of serum levels of the liver enzyme alanine transaminase (ALT). The ALT elevations were mostly transient and eventually subsided, but there remains a concern about the safety of a live dengue virus vaccine. Passive immunization with clinically acceptable dengue virus neutralizing antibodies provides an attractive alternative to prevention of dengue virus infection. Highly efficient neutralizing antibodies might also be useful for consideration as an effective therapy for severe dengue. Recently, phage display of combinatorial antibody libraries has allowed isolation of antibodies against important viral pathogens from human or non-human primates (8, 12, 41, 49, 56, 63). In the current study, we employed this technique to identify a panel of chimpanzee Fab antibodies against DENV-4. One of these Fab antibodies neutralized DENV-4 efficiently by an *in vitro* assay and was combined with human sequences to convert it to the whole IgG1 antibody. The humanized chimpanzee IgG1 antibody produced in CHO cells neutralized DENV-4 efficiently.

Materials and Methods

Preparation of dengue serotype 1-4 viruses.

Mosquito C6/36 cells were grown in MEM supplemented with 10% fetal calf serum. Confluent C6/36 cells were infected with DENV-4 at 0.1 moi in MEM containing 2% fetal calf serum and incubated at 28 C. The medium from the infected cells was harvested at 7 days and again at 10 days. It was clarified by centrifugation at 3,000 rpm in a JA10 rotor (1,000 g) and then centrifuged at 9,000 rpm in a JA10 rotor (15,000 g) overnight. The DENV-4 pellet was re-suspended in phosphate buffered saline (PBS) for phage panning and for ELISA. In addition, DENV-4 grown in C6/36 cells in serum-free medium (VP-SFM, Gibco) was directly used for panning and for ELISA. DENV-1 (Western Pacific strain), DENV-2 (prototype New Guinea C strain) and DENV-3 (strain H87) were prepared in serum-free medium from infected simian Vero cells.

Inoculation of chimpanzees with infectious DENV-4 RNA and with dengue serotype 1, 2, and 3 viruses.

Two dengue virus sero-negative chimpanzees, # 1616 and # 1618, were intrahepatically inoculated with infectious RNA transcripts made from the full-length cDNA clone of DENV-4 strain 814669 (36). A blood sample was collected weekly from each animal for analysis of the serum ALT levels and for analysis of antibodies to DENV-4. Eleven weeks after DENV-4 RNA inoculation, bone marrow was aspirated from the iliac crest of each chimpanzee and a combinatorial antibody library (designated library D4) was constructed. Eight-and-half months after inoculation with DENV-4 RNA, each of the chimpanzees was inoculated subcutaneously with a mixture of DENV-1, DENV-2 and DENV-3, each at 10^6 plaque forming units (pfu), in 1 ml of minimum essential medium (MEM) (Gibco) plus 0.25% human serum albumin. Six weeks after inoculation with the dengue virus mixture, serum samples were collected for analysis of antibody response. Twelve weeks after inoculation with DENV-1, DENV-2 and DENV-3, bone marrow was aspirated again and a second antibody library (designated library D1-4) was constructed. Both libraries were prepared from bone marrow of chimpanzee #1618, which developed slightly higher antibody titers against DENV-1, DENV-2 and DENV-3 than did chimpanzee #1616.

Construction of γ1/κ chimpanzee Fab antibody libraries

The lymphocytes from bone marrow were separated on a Ficoll-Paque gradient by centrifugation and aliquots of approximately 1×10^7 cells/ml in MEM containing 10% DMSO and 10% fetal calf serum were stored over liquid nitrogen. Total RNA was extracted from 3×10^7 lymphocytes using the RNA Extraction Kit (Stratagene) and mRNA was reverse-transcribed using oligo (dT) as primer (ThermoScript RT-PCR System, Invitrogen). The κ light chain DNA was amplified from the cDNA product by PCR using seven pairs of human κ light chain family-specific 5' primers and a 3' primer in the constant domain (4, 21, 49, 56). The γ1 heavy chain Fd cDNA was amplified using nine human γ1 heavy chain family-specific 5' primers plus a chimpanzee γ1-specific 3' primer (21, 56). A thirty-cycle PCR at 94 C for 15 s, 52 C for 50 s and 68 C for 90 s was performed with AmpliTaq DNA polymerase (Perkin Elmers).

Cloning of the chimpanzee κ light chain and γ1 heavy chain DNA fragments into the pComb 3H phage display vector was performed as described (4, 63). Briefly, amplified κ light chain DNA fragments were pooled, digested with Sac I and Xba I, and then cloned into pComb 3H (49) by transformation of electro-competent *E. coli* XL-1 Blue (Stratagene). Plasmid containing the γ1 light chain DNA inserts was prepared from *E. coli* transformants and then cleaved with Spe I and Xho I for insertion with amplified γ1 heavy chain DNA fragments cleaved with the same enzymes. The plasmid containing both the heavy chain and the light chain DNA inserts was used for transformation of *E. coli* XL-1 Blue by electroporation. In both electroporation steps, the ligated DNA mixture yielded a library size of 1-3 $\times 10^8$ *E. coli* colonies.

Panning of phage library and isolation of DENV-4-specific soluble Fabs.

Construction of phage display libraries, recovery and transfer of Fab sequences, and identification of *E. coli* transformants expressing DENV-4-specific soluble Fabs were carried out as described (21, 56). Briefly, approximately 10^8 transformants were grown in 2YT broth containing 1% glucose, 10 μg/ml tetracycline and 100 μg/ml ampicillin for 3 hr at 37C. The bacterial culture was then infected with helper phage VSC M13

(Stratagene) at 50 moi to generate the phage library. The phage library D4 was panned by affinity binding on DENV-4 virions coated directly on an ELISA plate that was blocked with 3% nonfat powdered milk in PBS to reduce non-specific binding. The phage library D1-4 was panned by affinity binding on DENV-4 virions captured by a chimpanzee serum immobilized on an ELISA plate to minimize conformational changes of the DENV-4 antigenic structure. Following three cycles of panning, the selected phage mixture was used to infect *E. coli* XL-1 Blue and replicative form DNA (phagemid) was prepared. Phagemid was cleaved with Nhe I and Spe I, and re-circularized to remove the phage gene III portion of the fused Fab sequence. *E. coli* XL-1 Blue were transformed with the circularized DNA and colonies that yielded soluble Fab fragments reactive to DENV-4 virus were screened by ELISA.

DNA sequencing of DENV-4 specific Fab clones

Plasmid from the selected *E. coli* transformants was initially analyzed by BstN1 digestion to identify Fab clones with distinct patterns. Sequence analysis of the Fab V_H and V_L DNA segments was performed on an automated DNA sequencer with the Fluorescence Dideoxynucleotide Terminator Cycle Sequencing Kit using Taq DNA polymerase (Perkin-Elmer). The following primers were used: 5' ACAGCTATCGCGATTGCAGTG (LC-1) and 5' CACCTGATCCTCAGATGGCGG (LC-4) for sequencing the V_L segment; 5' ATTGCCTACGGCAGCCGCTGG (HC-1) and 5' GGAAGTAGTCCTTGACCAGGC (HC-4) for sequencing both DNA strands of the V_H segment (21, 56). Software Vector NTI (InforMax) was used for sequence analysis. The DNAPLOT software program (MRC Center for Protein Engineering) was used to search for human immunoglobulin homologues in the data base.

Production and purification of Fab antibodies

Selected *E. coli* colonies were grown in 1 liter of L-broth containing 1% glucose and 100 µg/ml ampicillin and 10 µg/ml tetracycline to an early log phage (optical density at 600 nm approximately 0.2) at 30 C. The bacteria were then transferred to 2 liters of L-broth containing100 µg/ml ampicillin and 10 µg/ml tetracycline and grown at 30C in the presence of 0.1 mM of inducer IPTG for 5 h. The bacteria were pelleted and resuspended

in 20 ml of Extraction Buffer containing 50 mM sodium phosphate, 10 mM Tris-HCl, pH 8.0, 100 mM NaCl (Clontech), and 0.1 mM protease inhibitor AEBSF. After three cycles of freezing and thawing to release the soluble Fab product from the bacterial periplasm, the preparation was clarified by centrifugation at 10,000 rpm in a JA-20 rotor (10,000 g) for 60 min. The histidine-tagged Fab in the supernatant was purified through a column containing 1-ml bed volume of TALON Metal Affinity Resin (Clontech) using the pH elution procedure as suggested by the manufacturer. The Fab purity was verified by polyacrylamide gel electrophoresis using purified human IgG $F(ab')_2$ (Cappel) as a marker. The Fab concentration was determined colorimetrically using the BCA Protein Assay Kit (Pierce).

Biotinylation of purified Fab fragments and competition ELISA

Purified Fabs were biotinylated with EZ-Link NHS-LC-Biotin (Pierce) according to the procedure suggested by the supplier. After extensive dialysis against PBS, the biotin-labeled Fab was tested for binding to DENV-4 coated on wells of a microtiter plate. For competition ELISA, a fixed concentration of biotinylated Fab was mixed with a competing Fab in serial dilution and the mixture was added to the DENV-4-coated wells. Streptavidin-alkaline phosphatase was used for detection of biotinylated Fab bound to DEN4.

Radiolabeling of DENV-4 antigens and radio-immunoprecipitation

Infection with DENV-4 or recombinant vaccinia virus and subsequent radio-labeling of infected cells were performed as described earlier (18). Confluent Vero cells in a T-25 flask were infected with DENV-4 strain 814669 at 1 moi and incubated for 4 days at 37 C. Infected cells were rinsed once, starved for methionine in methionine-free MEM for 30 min and then labeled with ^{35}S-methionine at 150 µCi/ml (specific activity, 3000 Ci/mM). After a 6-h labeling period, cells were rinsed with cold PBS and lysed in 2 ml radio-immunoprecipitation assay (RIPA) buffer. Confluent CV-1 cells were infected with 5 moi of recombinant vaccinia virus vDENV-4 PrM (7) or vDENV-4 E (43) containing the full-length PrM or E coding sequence, respectively, for 15 h at 37 C. Infected cells were rinsed and starved for methionine in methionine-free MEM, placed in the labeling

medium for 2 h, and then lysed in RIPA buffer as described. A 20-μl labeled lysate of DENV-4- or recombinant vaccinia virus-infected cells was mixed with 10 μl of the Fab fragment to be tested and 70 μl RIPA buffer, incubated at 4 C overnight and then mixed with 2 μl of goat anti-human IgG $F(ab')_2$ antibody for 2 h. A 100-μl suspension of protein A-Sepharose beads was added to bind the radio-immune complexes. The Sepharose beads were collected by centrifugation and washed three times with RIPA buffer prior to separation by polyacrylamide gel electrophoresis. Radio-labeled protein bands on the dried gel were visualized by exposure to an X-ray film.

Construction of DNA recombinants and expression of full-length IgG1 in Chinese Hamster Ovary (CHO) cells.

The expression vector pFab CMV, kindly supplied by Dr. P. Sanna (Scripps Research Institute), was re-engineered for IgG1 production (Fig. 1). The vector contained a neomycin phosphotransferase gene (*neo*) located between the two hCMV promoters and a β-lactamase gene (*amp*) between the two poly A sites as mapped by restriction digestion and by sequencing. The *neo* and *amp* locations differed from the published map (54). A di-hydrofolate reductase (*dhfr*) gene together with the transcription signals was inserted at the unique Not I site in the original vector as the selecting marker and for gene amplification (64). The *dhfr* gene insert was the 1.4 kb DNA fragment from Pvu II/Afe I cleavage of pCDHC68B, kindly provided by Dr. K. Deen (2). The original plasmid vector contained an A at the last nucleotide position of the intron that precedes the C_H3 exon. This variant nucleotide was converted to G to allow proper RNA splicing for full-length IgG1 expression. The Fab 5H2 V_L DNA segment cleaved by Sac I and Xba I was first inserted into the expression vector. The resulting recombinant was then added with the V_H DNA segment cleaved by Xho I and Spe I, which was regenerated by PCR using the Fab 5H2 DNA template and appropriate primers. The chimpanzee-specific sequence in the hinge region together with the variant sequences introduced during plasmid construction were converted to the human hinge sequence using positive strand primer 5' GACAAAACTCAC<u>ACATGT</u>CCACCGTGCCCA, which introduced a Pci I site (underlined) with silent mutations (15, 59). Accordingly, the IgG1 antibody product would contain the chimpanzee V_H and C_H1 sequences and the entire human hinge, C_H2

and C_H3 sequences.

CHO/dhfr- (duk-) cells were purchased from American Type Culture Collection. Production of the whole IgG1 in CHO/dhfr- cells was carried out by transfection with RsrII-cleaved recombinant plasmid in the presence of Lipofectamine (Gibco). Two days after transfection, cells in a T25 flask were re-plated in Iscove's Modified Dulbecco medium (Gibco) supplemented with 10 % fetal bovine serum plus 10^{-7} M methotrexate in the absence of hypoxanthine/thymidine as selecting medium (13, 64). Transformed CHO cells resistant to 10^{-7} M methotrexate appeared approximately two weeks after transfection. Transformed CHO cells producing IgG1 in the medium were identified by ELISA and by PRNT following sub-cloning in a 96- or 24-well plate. Gene amplification was carried out step-wise by increasing methotrexate concentration to $2x10^{-7}$ M in the selecting medium. CHO cells that produced IgG1 at a high level were selected. The selected CHO cells were adapted to growth in suspension for IgG1 production in serum-free CD CHO medium (Gibco). Medium fluid was concentrated and the IgG1 product was purified through a protein-A affinity column. The full-length IgG1 5H2 antibody was compared with the Fab 5H2 fragment for DENV-4-binding affinity by ELISA. The equilibrium affinity constant (*Kd*) was calculated as the antibody concentration that gave 50% of maximum binding (38, 50).

Determination of DENV-4 neutralizing activity of Fab and whole IgG1 antibodies.

Affinity-purified Fab or full-length IgG1 antibodies were analyzed for DENV-4 neutralizing activity by a modification of plaque reduction neutralization test (PRNT), as described (47). Briefly, approximately 50 focus-forming units of DV-4 were mixed with a serial dilution of Fab or IgG1 antibodies in 250 ul of MEM. The mixture was incubated at 37 C for 30 min and then used for infection of Vero cell monolayers in a 24-well plate. The cells were overlaid with a semi-solid medium containing 1% Tragacanth gum (Sigma) and incubated at 37 C for 4 days. Foci of DENV-4 infected cells were visualized following immuno-staining with hyperimmune mouse ascites fluid (HMAF) and anti-mouse horseradish peroxidase conjugate (Pierce). The Fab or IgG1 concentration that produced 50% focus reduction was calculated. The neutralizing activity of the IgG1 antibody was tested against DENV-4 strain H241 isolated from the Philippines and two

Caribbean DENV-4 isolates, ie, strain 814669 and strain 341750.

Results

Chimpanzee antibody response to intrahepatic transfection with DENV-4 RNA and to subsequent inoculation with a mixture of DENV-1, DENV-2 and DENV-3 viruses.

Two chimpanzees (# 1616 and #1618) were intrahepatically transfected with the full-length RNA transcripts of cloned DENV-4 cDNA (36). Four weeks after inoculation, these chimpanzees showed transient mild serum ALT elevations and became sero-positive for DENV-4, indicating that both animals were infected (data not shown). At 9 weeks, the antibodies against DENV-4 reached a $PRNT_{50}$ titer of 1/ 992 and 1/1065, respectively. This level of neutralizing antibodies was comparable to that in rhesus monkeys infected with DENV-4 by a subcutaneous route (44). To increase the repertoire of dengue virus-specific antibodies, both chimpanzees were inoculated with a mixture of DENV-1, DENV-2 and DENV-3 viruses, each at 10^6 pfu/dose, ten-and-half months after DENV-4 RNA transfection. Both chimpanzees developed moderate to high $PRNT_{50}$ titers of antibodies against DENV-1, DENV-2 and DENV-3 (Table 1), indicating that the chimpanzees were infected with each of these viruses. Meanwhile, the $PRNT_{50}$ antibody titer against DENV-4 increased approximately 2 fold following infection with DENV-1, DENV-2 and DENV-3 viruses.

Chimpanzee $\gamma 1/\kappa$ combinatorial Fab antibody libraries

Two phagemid libraries were constructed from bone marrow mRNA of chimpanzee #1618: (A) Library D4 was prepared from the chimpanzee after intra-hepatic inoculation with DENV-4 RNA; and (B) Library D1-4 was prepared from the same animal after infection with a mixture of the other three dengue serotype viruses. Phage library D4 was panned three successive rounds against DENV-4 virions immobilized directly in an ELISA plate. After the third panning, plasmid was isolated and cleaved with Spe I and Nhe I for expression of soluble Fabs. Library D1-4 was panned three successive rounds against DENV-4 virions captured by chimpanzee antibodies coated on an ELISA plate. In this manner, possible conformational distortions of the DENV-4 virion surface due to direct coating on a solid phase might be minimized. Similarly, after the third panning,

plasmid was isolated and cleaved with Spe I and Nhe I for expression of soluble Fabs.

Identification and characterization of chimpanzee Fabs specific to DENV-4.

E. coli transformants were screened for production of soluble Fabs capable of binding to DENV-4. Plasmid containing the Fab insert was analyzed by digestion with BstN I in order to select distinct clones. Sequence analysis of the V_H and V_L DNA inserts identified Fabs 5A7, 3C1, 3E4, and 7G4 in library D4. Fabs 5H2 and 5D9, which varied in the V_L sequences but shared a nearly identical V_H sequence (a single amino acid difference in the FR3 region), were recovered from library D1-4 (Figure 2). The sequences in the heavy chain complementarity-determining region 3 (CDR3) (65), critical for antigen binding, showed a greater diversity than the sequences in other regions among these Fabs. A sequence similarity search of the available human immunoglobulin genes was conducted to determine the specific germ line origin of these chimpanzee Fab fragments. The chimpanzee V_H and V_L sequences and their most related human immunoglobulin genes of the germ line VH or Vκ families are shown (Table 2). These chimpanzee V_H or V_L sequences and their human homologues shared 88-95% identity excluding the CDR3 region.

Antigenic specificity of chimpanzee Fab monoclonal antibodies

First, the binding activity of the Fab antibodies to DENV-4 was analyzed by ELISA. All six selected Fabs showed strong binding to DENV-4 virions (Table 3). Chimpanzee Fab 1F2, which was selected from library D4 for its ability to bind anti-human $F(ab)'_2$ but not DENV-4, was used as the control. A cross-reactivity to DENV-1 or DENV-2 was detected for Fabs 5A7, 3E4 and 7G4. Other Fabs showed no detectible cross-reactivity to DENV-1, DENV-2 or DENV-3 virus. Radio-immunoprecipitation using a lysate of DENV-4 infected Vero cells was then performed to determine the antigen-binding specificity (Fig. 3A), Fab 5A7 selectively precipitated prM. All other Fabs precipitated both E and prM. The amount of prM relative to E precipitated varied, depending on the Fab. Radio-immunoprecipitation was again performed using labeled E or prM prepared individually in recombinant vaccinia virus-infected cells (Fig. 3B). Fabs 3E4 and 7G4 precipitated E, but not prM. Fab 3C1 precipitated neither E nor prM. Fab 5D9

precipitated E, but not prM, whereas Fab 5H2 precipitated E and a trace of prM. When the labeled antigens were mixed, co-precipitation of prM and E was again detected for Fabs 3E4, 7G4, 3C1 and 5H2.

Mapping Fab antibody binding sites on DENV-4 virions by competition ELISA

Biotinylated Fabs 3C1, 3E4, 7G4 and 5H2 were each tested for binding to DENV-4 in the presence of an unlabeled, competing Fab. Chimpanzee Fab 1F2, which did not bind DENV-4, was analyzed in parallel. Fab 5D9, which was nearly identical to Fab 5H2, was not tested. The result (Fig. 4, panels A-D) showed that binding of Fab 3C1 to DENV-4 was competed by Fab 5A7, but not by Fab 3E4, 7G4 or 1F2. Thus, the binding site on PrM for Fab 3C1 overlapped with that for Fab 5A7. Fab 3E4 and Fab7G4 also competed with each other for binding to DENV-4, indicating that their binding sites on E overlapped. The binding site on E for Fab 5H2 was unique, as binding competition with other Fabs was not observed.

DENV-4 neutralizing activity of Fab antibodies

Affinity-purified Fabs were used for $PRNT_{50}$ determination (Table 4). Similar to the Fab 1F2 control, prM-specific Fab 5A7 or 3C1 did not neutralize DENV-4. Fabs 3E4 and 7G4 exhibited a low neutralizing activity with a $PRNT_{50}$ titer at 91 ug/ml or greater. Importantly, Fab 5H2 and Fab 5D9 neutralized DENV-4 efficiently, with a $PRNT_{50}$ titer of 0.24 and 0.58 ug/ml, respectively.

Humanized chimpanzee full-length IgG1 antibodies produced in CHO cells.

Production of full-length antibodies from the Fab $\gamma 1/\kappa$ sequences was achieved with expression vector pFab CMV-dhfr, which provides a portion of the hinge and the entire C_H2 and C_H3 sequences of the human gamma-1 heavy chain (Fig. 1). A *dhfr* gene was inserted into the vector for selection of antibody-producing CHO cells with methotrexate and for gene copy amplification. Other modifications of the expression vector included conversion of the chimpanzee-specific hinge sequence to the human counterpart and an A to G substitution at the last nucleotide of the intron between CH_2 and CH_3 exons of the heavy chain sequence (see Materials and Methods). Thus, the product was a full-length,

chimeric human-chimpanzee (humanized) IgG1 antibody. Fab 5H2 was chosen for conversion to the whole IgG1 antibody. The full-length IgG1 5H2 was secreted into the culture medium of the transformed CHO cells and the yield of the affinity-purified product was approximately 1.8 mg per liter. Affinity-purified IgG1 5H2 was compared with Fab 5H2 for binding affinity to DENV-4 by ELISA. The IgG1 5H2 and Fab 5H2 had equilibrium affinity constants (*Kd*) of 0.24 nM and 0.42 nM, respectively. IgG1 5H2 neutralized three DENV-4 strains from two geographic regions *in vitro* at a similar high $PRNT_{50}$ titer of 0.03-0.05 ug/ml (Fig. 5). Humanized IgG1 5H2 represents the first DENV-4-neutralizing monoclonal antibody of primate origin.

Discussion

The last few decades have seen the isolation and characterization of a large number of murine monoclonal antibodies against the four dengue viruses and other arthropod-borne flaviviruses. The clinical utility of these murine monoclonal antibodies is limited, because of their propensity to induce an antibody response in humans. To develop a strategy of passive immunization against dengue, we turned to antibodies from chimpanzees, which are closely related to humans and can be experimentally infected with dengue viruses. The current study represents the first successful recovery of chimpanzee Fab monoclonal antibodies against the dengue virus by combinatorial cloning.

Analysis of the series of Fab antibodies against DENV-4 recovered by combinatorial cloning suggested a pattern of chimpanzee antibody response to intrahepatic infection with the infectious DENV-4 RNA transcripts. As in dengue virus infection of mice, both PrM-specific (Fab 5A7) and E-specific (Fabs 3E4 and 7G4) antibodies were identified in he chimpanzee. Interestingly, both Fab 3E4 and Fab 7G4 antibodies co-precipitated prM and E, when the two antigens were mixed. Fab 3C1 also co-precipitated prM and E, but did not precipitate either when these antigens were present individually. These results suggest that these Fabs recognized either PrM or E in the prM-E heterodimer. These chimpanzee Fab antibodies may be useful for analysis of PrM-E interactions and the antigenic structure of dengue virus. Nevertheless, their DENV-4-neutralizing activity was low or not detected and they are not likely to be effective against the virus.

Our goal of recovering antibodies highly efficient for neutralizing DENV-4 was achieved by repertoire cloning of chimpanzee bone marrow following multiple dengue virus infections. In this case, DENV-4 virions captured by polyclonal antibodies immobilized on plates were used for phage panning. The panning modification might better preserve the native conformation of the DENV-4 antigenic structure. This experiment yielded Fabs 5H2 and 5D9 that neutralized DENV-4 efficiently at a $PRNT_{50}$ titer in the range of 0.2-0.6 ug/ml. Both Fabs shared a nearly identical V_H sequence, but varied in the CDR1, CDR2 and other regions of their V_L sequences. These differences in the V_L and V_H sequences could explain the observation that Fab 5H2 co-precipitated E and prM, whereas Fab 5D9 precipitated only E. Importantly, both Fabs neutralized DENV-4 efficiently at a $PRNT_{50}$ titer in the range of that of human Fab antibodies against the respiratory syncytial virus (12), Ebola virus (41) or human immunodeficiency virus (5) selected by phage display.

Fab antibody fragments have a rapid clearance rate in humans, and therefore are not directly useful clinically. Conversion of the Fab fragments to their whole IgG1 antibody molecules was achieved using expression vector pFab CMV-dhfr, which provided a portion of the hinge and the complete C_H2 and C_H3 heavy chain sequences from a human germ-line DNA segment. A dihydrofolate reductase gene was inserted in the expression vector to increase the IgG1 antibody production. The chimpanzee-specific sequence found in the hinge region was also converted to the human sequence. The humanized IgG1 5H2 had at least equal binding affinity for DENV-4, if not higher than Fab 5H2, as measured by equilibrium affinity constants. Importantly, the humanized antibody IgG1 5H2 exhibited a $PRNT_{50}$ titer of 0.03-0.05 ug/ml, approximately 8 fold more efficient than that of the Fab fragment against DENV-4.

Polyclonal antibody preparations against Caribbean DENV-4 isolates, including strain 814669, have been shown to neutralize DENV-4 H241 isolated from the Philippines less efficiently than the homologous DENV-4 strains, suggesting that there are antigenic variations among DENV-4 strains with different geographical origins (30). Sequence analysis also indicates that there is a significant genetic variation among DENV-4 isolates from different geographic regions (37). Thus, it is significant that IgG1 5H2 was able to neutralize geographically diverse DENV-4 isolates at a similar high titer.

The DENV-4-neutralizing activity of IgG1 5H2 was approximately 6-10 fold higher than the IgG monoclonal antibody against the Ebola virus (41) and 40-60 fold higher than the humanized mouse antibody (MEDI-493) against respiratory syncytial virus (33). The CHO cell line obtained in this study produced the humanized chimpanzee antibody IgG1 5H2 at approximately 1.8 mg per liter. Increased production of this antibody in other mammalian cell systems should be possible.

A computer search revealed that the amino acid sequences of V_H and V_L segments of these Fab antibodies shared a strong homology, ranging from 88-95 %, with the sequences of their human immunoglobulin homologues. In particular, the Fab 5H2 $\gamma 1$ heavy chain and κ light chain sequences shared 89% and 94% sequence similarity to the human germ line IgG gene homologues, excluding the CD3 region (48, 60). Further, there was only one amino acid difference in the C_H1 or C_L1 region between chimpanzee and human sequences (data not shown). The high level of antibody sequence similarity and a number of other observations addressing this issue suggest the possibility that chimpanzee antibodies may be administered directly to humans without further modifications to humanize these reagents (16, 17). Experimental data available indicate that little immunogenicity is seen when components of human antibodies are introduced into chimpanzees (46).

The cause of severe dengue sometimes associated with secondary dengue virus infection remains controversial. According to one hypothesis, in a secondary infection dengue virus could form a complex with a sub-neutralizing level of cross-reactive antibodies produced during the primary infection, leading to an enhanced uptake and replication in susceptible mononuclear cells via their Fc receptors (23). Several classes of FcR receptors have been identified on the cell surface and their interacting amino acids in the respective IgG have been carefully mapped (1, 10). It is now possible to ablate the FcR receptor binding sequences in the antibody molecules and to test their activity for enhancing dengue virus replication *in vitro* (3, 55, 57). Humanized chimpanzee IgG1 monoclonal antibodies lacking the FcR1 binding sequences should permit a critical test of the hypothesis. For clinical application, it is important that these humanized chimpanzee antibodies not enhance dengue virus replication in human monocytes or other FcR receptor-bearing cells.

Since there are four dengue virus serotypes, monoclonal antibodies against each of the remaining three dengue serotypes will be required for effective prevention of dengue infection using this approach. It should be possible similarly to identify Fab antibodies from the infected chimpanzees that efficiently neutralize each of the other three dengue virus serotypes. Accordingly, a panel of humanized chimpanzee monoclonal antibodies that most efficiently neutralize these dengue viruses will be prepared and evaluated for protection against dengue infection in animal models and ultimately in humans.

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Figure Legends

Figure 1. A map of pFab CMV-dhfr vector for expression of full-length IgG1 in CHO cells and structure of the IgG1 light chain and heavy chain DNA inserts. (A) Locations of the various genes present in the expression vector- *dhfr*, dihydro-reductase; *neo*, neomycin phosphotransferase; hCMV, human CMV promoter; LC, light chain DNA; pA, polyA addition signal; *amp*, pBR 322 β-lactamase and the DNA replication origin; HC, heavy chain DNA. Arrows indicate transcription direction. (B) Structure of the humanized IgG1 light chain and heavy chain genes under the control of a hCMV early promoter. V_L and C_L1 are the light chain hyper-variable region and constant region 1, respectively; V_H, C_H1, hg, int-1, C_H2, int-2, and C_H3 represent the heavy chain hyper-variable region, constant region 1, hinge, intron-1 (118 nucleotides), constant region 2, intron-2 (97 nucleotides) and constant region 3 in that order. The dark-shaded regions are human IgG1 sequences and the medium-shaded regions represent chimpanzee IgG1 sequences. The selectable *neo* and *dhfr* genes (light-shaded) are flanked by a β-globin promoter and a poly A addition site.

Figure 2. Alignment of amino acid sequences among DENV-4-specific Fab monoclonal antibodies. The amino acid sequences of the six chimpanzee Fab monoclonal antibodies recovered by repertoire cloning were compared. (A) Sequences of V_L light chain segments. (B) V_H heavy chain segments. The framework regions (FR1-4) and complementarity-determining regions (CDR1-3) are shown. The dash symbol is placed where an amino acid deletion occurred and an identical amino acid is represented by a comma.

Figure 3. Analysis of antigenic specificity by radio-immunoprecipitation.
(A) ^{35}S-methionine labeled lysates of DENV-4-infected Vero cells were precipitated with the various Fab preparations indicated. (B) ^{35}S-methionine labeled lysates were prepared from Vero cells infected with vaccinia virus recombinant vDENV-4 prM or vDENV- E containing the full length coding sequence of prM or E, respectively. E+prM: precipitations using a mixture of both lysates. HMAF: precipitation using hyper-immune mouse ascitic fluid raised against DENV-4.

Figure 4. Epitope analysis of chimpanzee Fab antibodies against DENV-4 by competition ELISA. Selected Fabs were affinity purified, biotinylated and used for analysis of binding reactivity to DENV-4 virions by competition ELISA in the presence of competing, unlabeled Fabs. (A) Biotinylated Fab 3C1; (B) Biotinylated Fab 3E4; (C) Biotinylated Fab 7G4; (D) Biotinylated Fab 5H2. Chimpanzee Fab 1F2, which did not bind to DENV-4, was used as a negative control. The numbers on the Y-axis are OD readings and the X-coordinate represents reciprocal dilutions of the competing Fabs.

Figure 5. *In vitro* neutralization of DENV-4 strains by humanized chimpanzee antibody IgG1 5H2. Full-length antibody IgG1 5H2 was concentrated from the culture medium of transformed CHO cells selected with $2x10^{-7}$ M methotrexate and then affinity-purified through a protein-A column. The neutralizing activity of the antibody preparation was tested by PRNT against DENV-4 H241 isolated in the Philippines and DENV-4 814669 and DEV-4 341750, isolated in the Caribbean.

Fig. 1A

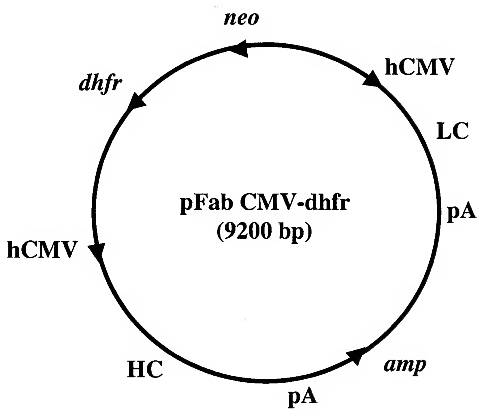


Fig. 1B

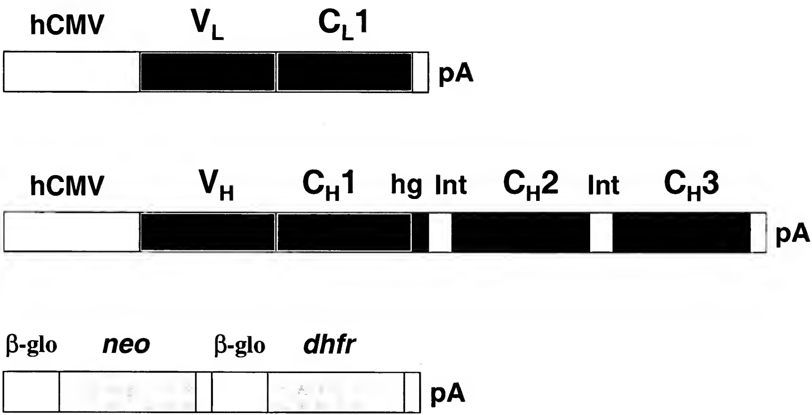

hCMV
V$_L$
C$_L$1
pA
hCMV
V$_H$
C$_H$1
hg
Int
C$_H$2
Int
C$_H$3
pA
β-glo
neo
β-glo
dhfr
pA

Fig. 2

(A)

	FR1	CDR1	FR2	CDR2
5A7	--ELTQGPATLSLSPGERATLSC	RAGQSLDS----SLLS	WYQQKPGQAPRLLMY	DASTRAP
3C1	ELQM,,S,S,,,A,V,D,V,VT,	,,SED,N-----KW,A	,,,,,,,K,,K,,I,	K,,SLES
3E4	--,,,,S,LS,PVTL,QP,SI,,	,SS,N,VHSDGNTY,,	,I,,R,,,P,,,,I,	KV,N,DS
7G4	--,,,,S,S,,,A,V,D,V,IT,	,,S,GIS-----,W,A	,,,,,,,K,,KF,I,	K,,SLES
5H2	ELQM,,S,SS,,A,V,D,V,IT,	,,S,DIS-----IR,N	,,,,,,,K,,K,,I,	,,,,LES
5D9	--,,,,S,SS,,A,V,D,V,IT,	,,S,GIS-----NR,N	,,,,,,,,G,KF,,,	,,,SLVS

	FR3	CDR3	FR4
5A7	GVPARFSGSGSGTDFTLTISSLQPEDFAVYY	CQQHYNLPRT	FGQGTKLEIKRT
3C1	,,,S,,,,,,,,,E,,,,,,,,,,D,,,T,,	,,,YQSY,Y,	,,P,,,,,,,,,
3E4	,,,D,,,,,,A,,,,,,K,TRVEA,,VGL,,	,V,GVQF,I,	,,,,,,,,,,,,
7G4	,,,S,,,,,,,,,E,,,,,,,,,,D,,,T,,	,,,YGSY,L,	,,P,,,VD,,,,
5H2	,,,S,,,,,,,,,,,,,,,,,,,,,,,,T,,	,,,FNSY,L,	,,G,,,V,,,,,
5D9	,,,S,,,,,,,,,,,,,,,,,,,,,,,,,,,	,,,FNSY,L,	,,G,,,,,,,,,

(B)

	FR1	CDR1	FR2	CDR2
5A7	EVQLLES-GGGLVQPGGSLRLSCAASGVTFS	SYWMH	WVRQAPGKGLEWV	SRINSDGSSTNYADSVEG
3C1	,,,,,,,-,AEVKK,,,,VKV,,KV,,G,L,	,,GIS	,,,,,,,Q,P,,M	GV,IPIRGTA,,,QKFQ,
3E4	,,,,,,QS,AEVKK,,,,VKV,,KV,,G,,,	RNPIS	,,,,,,,Q,,,,M	,V,VPIVGT,KH,QKFQ,
7G4	,,,,,,,-,,,,,,,,,,T,,,,,,F,,,	,,,,,	,,,,,,,,,P,,,	AL,KK,,,EKY,,E,,K,
5H2	,,,,,,,-,P,,,K,SET,S,T,TV,,GSI,	DFYWS	,L,,S,,,,,,,I	GYAH,RV,-AY,NP,LKS
5D9	,,,,,,,-,P,,,K,SET,S,T,TV,,GSI,	DFYWS	,L,,S,,,,,,,I	GVAH,RV,-AY,NP,LKS

	FR3	CDR3	FR4
5A7	RFTISRDNAKNTLYLQMNSLRAEDTAVYYC	SRGGLWDWSPRRIEETKTPFDY	WGQGTLVTVSS
3C1	,V,YTA,ESTS,V,MELS,,,S,,,,,,,,	AT-------G,,GRYPTGS,,,	,,,,A,,,,,,
3E4	,V,,TA,ESTS,A,MELS,,,S,,,,,,,,	AT-------Y,,YADVSSYSE,	,,,,,,,,,,,
7G4	,,,,,,,,,,,S,,,,,,,,,,,,,,,,,,	T,----- RITTLTVISDA,,I	,,,,,M,,,,,
5H2	,V,,,V,TS,,Q,S,RLSAVT,A,,,L,,,	A,-----QGTGTTGVSED,F,L	,,,,,K,I,,L
5D9	,V,,,V,TS,,Q,S,RLSAVT,A,A,L,,,	A,-----QGTGTTGVSED,F,L,	,,,,K,I,,L,

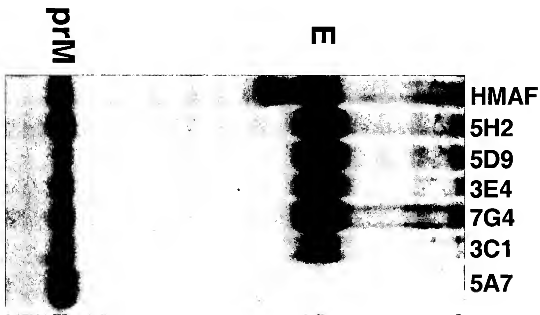


Fig. 3A

Fig. 3B

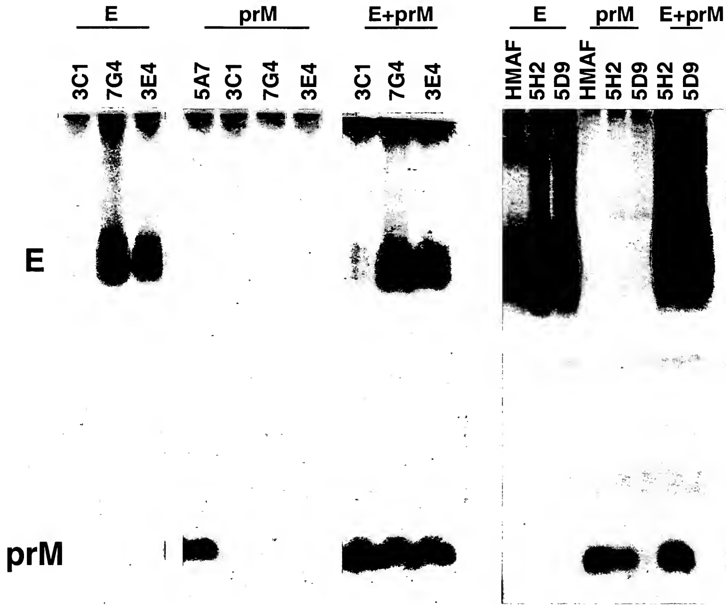

E
prM
E+prM
E
prM
E+prM
3C1
7G4
3E4
5A7
3C1
7G4
3E4
3C1
7G4
3E4
HMAF
5H2
5D9
HMAF
5H2
5D9
5H2
5D9
E
prM

Fig. 4

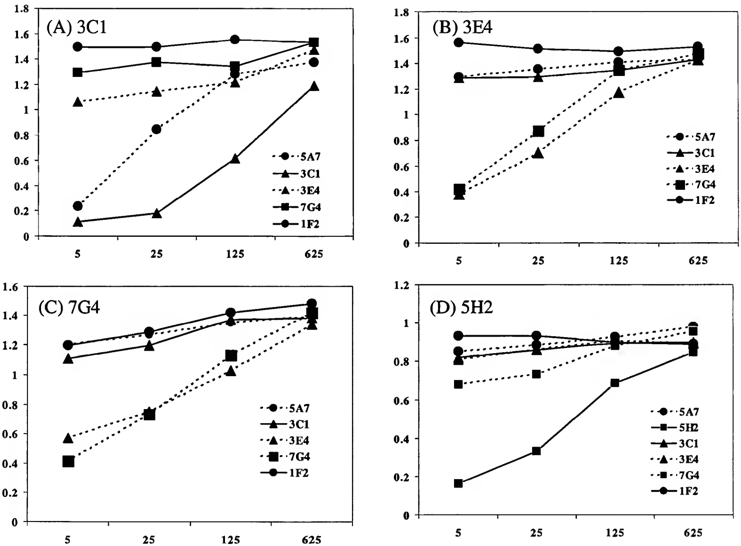
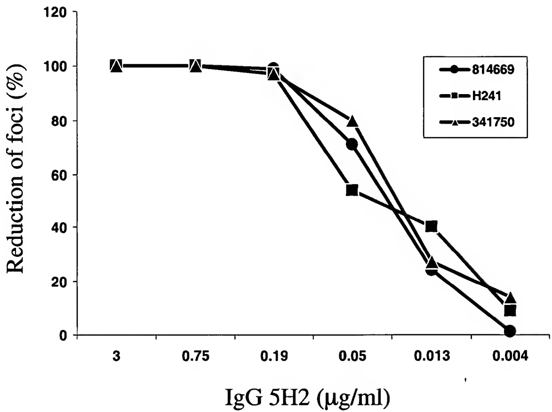

(A) 3C1
(B) 3E4
(C) 7G4
(D) 5H2
5A7
5H2
3C1
3E4
7G4
1F2
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
5
25
125
625

Fig. 5


120
100
80
60
40
20
0
Reduction of foci (%)
814669
H241
341750
3
0.75
0.19
0.05
0.013
0.004
IgG 5H2 (µg/ml)